

Abstract—Adult horseshoe crabs (*Limulus polyphemus*) are the preferred bait in the U.S. east coast whelk pot fishery, but their harvest is being restricted because of severe population declines in the Chesapeake and Delaware bays. To identify other baits, the activity in the pallial nerve of whelks was determined during exposure of the osphradium to odorant solutions prepared from horseshoe crab eggs, horseshoe crab hemolymph, and hard clam (*Merccenaria mercenaria*) tissue. All three elicited significant responses; bait based on them may provide an alternative to the use of adult horseshoe crabs, although extensive behavioral testing remains to be done. Channeled whelk did not respond to molecular weight fractions (>3 kDa and <3 kDa) prepared from horseshoe crab egg odorant solutions but did respond when the molecular weight fractions were recombined. Whelks appear to have broadly tuned chemoreceptors and manufactured baits may need to mimic the complex mixture of odorants derived from natural sources.

Activity in the pallial nerve of knobbed (*Busycon carica*) and channeled (*Busycotypus canaliculatum*) whelks recorded during exposure of the osphradium to odorant solutions

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Knobbed and channeled whelks (*Busycon carica* and *Busycotypus canaliculatum*, respectively) are common in inshore areas of the east coast of the United States from Georgia to Massachusetts (Walker, 1988) and form the basis of a substantial commercial fishery. The currently preferred bait in the pot fishery is adult horseshoe crabs (*Limulus polyphemus*) (Ferrari and Targett, 2003). The directed harvesting for bait has contributed to severe population declines of horseshoe crabs in the Chesapeake and Delaware bays (Berkson and Shuster, 1999) and increasingly stringent catch restrictions are being implemented. These restrictions include a moratorium on horseshoe crab harvest from Delaware Bay (issued by the Delaware Department of Natural Resources and Environmental Control and the New Jersey Department of Environmental Protection in 2006). These restrictions are being promulgated in large

measure because horseshoe crab eggs are a primary food source for migratory shorebirds, such as the red knot (*Calidris canutus*), during their northward spring migrations (Tsipoura and Burger, 1999; Weidensaul, 2006). Because of a continuing decline in the breeding population of the red knot (Morrison et al., 2004), the species has been proposed for inclusion on the Endangered Species List (Weidensaul, 2006).

Work is underway to create alternative bait for the whelk pot fishery (Ferrari and Targett, 2003). As part of these efforts, the sensitivities of knobbed and channeled whelks to odorant solutions prepared from horseshoe crab eggs, horseshoe crab hemolymph, and hard clam (*Merccenaria mercenaria*) tissue were examined. Odorant solutions prepared from horseshoe crab eggs, and molecular weight fractions of these, were tested because they have been reported to

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be attractive to mud snails (*Ilyanassa obsoleta*), and because horseshoe crab eggs have been proposed as a source of compounds for inclusion in manufactured bait (Ferrari and Targett, 2003). Horseshoe crab hemolymph was tested as a potential source of attractant for inclusion in replacement bait because it is a readily available natural product. It is a waste product produced during the preparation of *Limulus* amoebocyte lysate (LAL), a clotting agent used worldwide to test for bacterial contamination of pharmaceuticals and implantable medical devices. More importantly, when handled properly, horseshoe crabs used for hemolymph collection show low rates of postrelease mortality (Walls and Berkson, 2000). A patent has been issued for hemolymph-based bait for whelks (U.S. Patent 6391295), but we are unaware of any published studies documenting its efficacy in the whelk pot fishery. Odorant solutions prepared from hard clam tissue were tested because bivalve mollusks are a common prey item for whelks (Walker, 1988).

Electrophysiological techniques were used to test each proposed bait, rather than behavioral methods, because the former allows precise control of stimulus parameters and the rapid assay of various compounds and concentrations on individual animals. Electrophysiological techniques, moreover, have been used for many years to investigate successfully chemosensory questions in both aquatic invertebrates (e.g., Borroni et al., 1986; Kamio et al., 2005) and vertebrates (e.g., Hara, 1975; Wilson, 2004). Behavioral methods, such as flume choice (Y-maze) experiments, can also be difficult with whelks. A large cross-sectional area within a flume is necessary to accommodate adult whelks. This large cross-sectional area requires a high-volume flow rate and concomitantly large volumes of odorant solution to achieve a detectable concentration over the long time periods necessary for whelk to respond behaviorally (Ferner and Weissburg, 2005); large volumes of odorant solutions can be difficult and costly to produce (Ferrari and Targett, 2003; Ferner and Weissburg, 2005). Electrophysiological techniques, however, cannot differentiate between attractive and repulsive odors and must subsequently be paired with behavioral studies.

Activity in the pallial nerve was recorded while odorant solutions were applied directly to the osphradium. The pallial nerve connects the osphradium to the supraesophageal ganglia, which is part of the central nervous system (Alexander, 1970). The osphradium is considered to be the primary chemosensory organ of prosobranch mollusks because its leaf-like structure bears a strong resemblance to the nasal rosettes of aquatic vertebrates (Hansen and Reutter, 2004), and because its location at the base of the incurrent siphon maximizes exposure to odorants (Bailey and Laverack, 1966; Emory, 1992). It should be noted, however, that the osphradium is not the only chemosensory organ in gastropod mollusks. The rhinophores may also provide sensory information allowing gastropod mollusks to track odor plumes (Levy et al., 1997; Rahman et al., 2000; Ferner and Weissburg, 2005). Indeed, the rhino-

phores are considered a primary chemosensory organ in nudibranch mollusks (Alexander, 1970; Wedemeyer and Schild, 1995). A record of activity in the pallial nerve during exposure of the osphradium to odorant solutions derived from various sources therefore provides a robust method to determine whether a specific odorant solution is detectable by whelks and is a candidate for subsequent behavioral testing.

Materials and methods

Knobbed and channeled whelks were obtained from local processing plants or collected from estuaries behind the barrier islands on the eastern shore of Virginia. They were maintained at the Virginia Institute of Marine Science (VIMS) (Gloucester Point, VA) and also at the VIMS Eastern Shore Laboratory (Wachapreague, VA) in tanks supplied with running water drawn directly from the mouth of the York River or the Virginia eastern shore estuaries, respectively.

Horseshoe crab eggs were obtained from beaches bordering the Chesapeake and Delaware Bays, sealed in sterile 50-mL plastic tubes, and stored frozen. Hard clams were obtained from stocks maintained by the VIMS Eastern Shore Laboratory and only fresh tissue was used. Stock odorant solutions were prepared according to methods described by Ferrari and Targett (2003). In brief, horseshoe crab eggs were mixed (1:2 egg to liquid volume) with aerated, filtered, and ultraviolet sterilized water (FSW) drawn from the same sources supplying the holding tanks. The tissue was then crushed with a clean mortar and pestle and the mixture was allowed to sit overnight at 4°C. It was then centrifuged to remove cellular debris and sand. Clam tissue was treated likewise. In addition, horseshoe crab extract solution was prepared as described above, with the exception that 50 mM Tris buffer solution (pH 7.5, a mixture of Trizma HCl and Trizma Base, Sigma Chemical Co, St. Louis, MO) was used instead of FSW. Two molecular weight fractions (>3 kDa and <3 kDa) were then generated by using stirred cell ultra-filtration with YM-3 membranes (Millipore Inc., Billerica, MA). The molecular weight fractions were tested individually on channeled whelk, and after they were recombined. The stock odorant solutions were diluted with FSW 1:10³, 1:10⁴, 1:10⁵, and 1:10⁶ (volume to volume) immediately before experiments. Horseshoe crab hemolymph (free of any anticoagulants or preservatives) was obtained from Wako Chemicals (Cape Charles, VA) and stored at 4°C. It was likewise diluted (1:100, 1:133, 1:200, and 1:400) with FSW immediately before use.

During experiments, an individual whelk was presented with at least four concentrations of an individual odorant solution, plus controls consisting of FSW or a 50 mM Tris buffer solution in random order. The limited funding available for this project precluded the testing of all odorants on both species of whelks. The specific odorants presented to each whelk species are summarized in Table 1.

Table 1

Summary of odorant solutions derived from various sources tested on knobbed (*Busycon carica*) and channeled (*Busycotypus canaliculatum*) whelks. The odorant solutions that were tested on a species are indicated by an "X". FSW = aerated, filtered, and ultraviolet sterilized water.

	Knobbed whelk	Channeled whelk
Horseshoe crab (<i>Limulus polyphemus</i>) eggs extracted with FSW	X	X
Hard clam (<i>Mercenaria mercenaria</i>) tissue	X	
Horseshoe crab hemolymph	X	X
Horseshoe crab eggs extracted with 50 mM Tris buffer solution, <30 kDa and >30 kDa molecular weight fractions of horseshoe crab eggs produced by ultrafiltration, and the molecular weight fractions recombined		X

Quantifying activity in the pallial nerve in response to odorant solutions required that the whelk be removed from its shell, the pallial nerves exposed, and the osphradium isolated. To remove the animal from its shell, the apex of the shell was cut away at the spire, the columella muscle detached from the central column, and the animal gently pulled through the opercular opening. The individual was then immediately submerged in a large dish of aerated FSW. The viscera and the majority of the foot were removed and the mantle cavity opened with a dorsal incision starting medially between the cephalic eye stalks. The preparation was pinned to a Sylgard® (Dow Corning Corp., Midland, MI) lined Petri dish such that the body cavity was open and the buccal mass held out of the way. The overlying connective tissue was carefully dissected to expose the circumesophageal ganglia, specifically the supraesophageal ganglion, and the Petri dish holding the preparation was moved to a second plastic chamber (filled with FSW) mounted under a dissecting microscope. A modified 10-mL plastic syringe barrel with a small amount of quick-setting silicon elastomer (Kwik-Cast®, World Precision Instruments, Sarasota, FL) around the base was placed over the osphradium to isolate it from the fluid in the chamber containing the animal (Fig. 1A).

The odorant delivery system consisted of a glass reservoir (filled with FSW) and polyethylene and stainless steel tubing (Fig. 1A). FSW was continuously delivered to the osphradium chamber by gravity at approximately 6 ml/min. Odorant solutions were switched into the delivery system without a change in flow rate by using a three-way stopcock. The 10-mL volume odorant solution was therefore delivered to the osphradium chamber within approximately 90 seconds, and then flushed out of the osphradium chamber by switching the flow back to FSW. Because the volume of liquid in the osphradium chamber was maintained as close to 2 mL as possible, peak stimulus concentration in the osphradium chamber reached approximately 60% of the original stimulus concentration (Fig. 1B) (Steffensen, 1989).

To record activity in the pallial nerve associated with the exposure of the osphradium to odorant solutions, the nerve was cut and the afferent end was drawn into

a suction electrode (Fig. 1B). The resulting signal was amplified (80 dB gain) and filtered (10 Hz high pass and 1 k Hz low pass) by using a DAM-50 amplifier (World Precision Instruments, Sarasota, FL). The signal was further conditioned to remove 60 Hz noise with the use of a Humbug® active electronic filter (Quest Scientific, North Vancouver, B.C., Canada), and then displayed on a digital storage oscilloscope. The signal was digitized (at 1 kHz sampling rate) with a USB analog to digital I/O interface (model 1208LS, Measurement Computing, Middleboro, MA). The digital data were filtered (5 Hz high pass and 75 Hz low pass filters), processed, recorded, and displayed on a computer screen by using a custom designed computer program developed within DasyLab (version 7.0, National Instruments Corp., Austin, TX).

It was not practical to quantify responses by discrimination and by counting single nerve spikes because the recordings contained a broad amplitude signal due to activity in multiple nerve fibers, and because of the long intervals of activity in response to odorant solutions (Fig. 2). Instead, the differentiation and integration module within DasyLab was used to generate an output value proportional to the integral of the filtered nerve signal (Fig. 2B). The module was programmed to integrate over 10.2-second intervals (i.e., 20 data blocks, as defined within the DasyLab program) to reset the output to zero, and then resume integrating. The digital system was thus functionally equivalent to an analog electronic "leaky RC integrator" circuit commonly used to measure the magnitude of nerve activity (e.g., Hara, 1975; Kamio et al., 2005).

For each trial, data were recorded for 12 minutes: 6 minutes before the introduction of an odorant solution into the osphradium chamber, and 6 minutes after. This recording interval was chosen on the basis of long integration times reported for the chemosensory abilities of whelks (Ferner and Weissburg, 2005) and other mollusks (Murphy and Hadfield, 1997). An additional 3 minutes were allowed between odor trials to ensure that the odorant solution was flushed from the osphradium chamber and that the activity in the pallial nerve had returned to prestimulus levels (Fig. 2). Only data

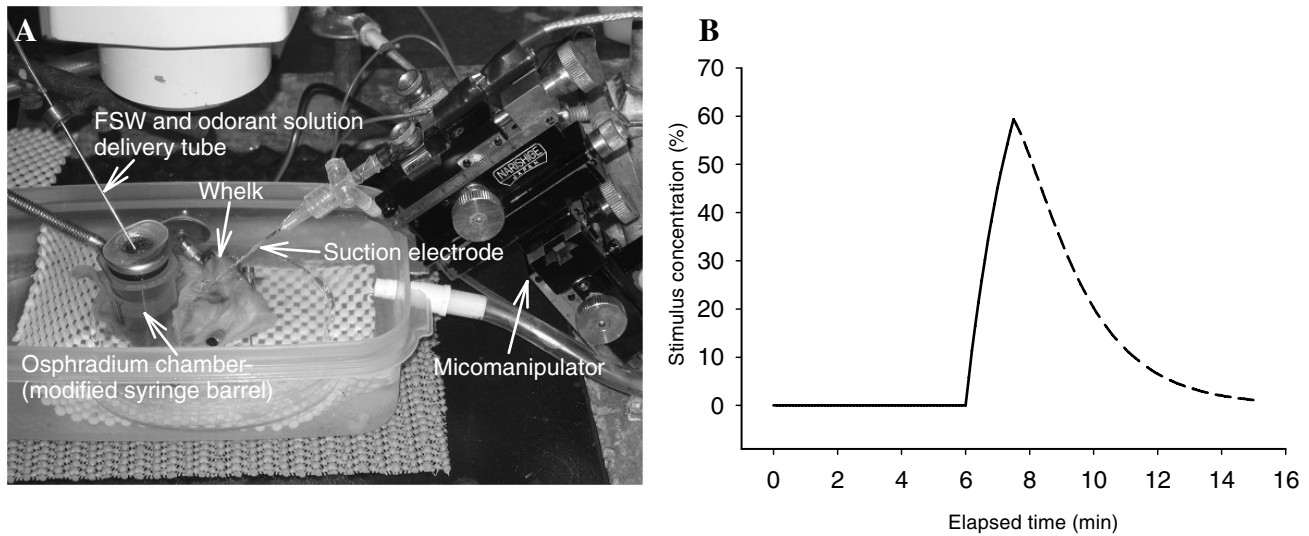


Figure 1

(A) Experimental setup showing a whelk, the osphradium chamber (modified syringe barrel), the aerated, filtered, and ultraviolet sterilized water (FSW) and odorant solution delivery tube, and the micromanipulator holding the suction electrode. (B) The predicted growth (solid line) and decay (dashed line) of the concentration of stimulus within the osphradium chamber over the course of a 15-minute trial, expressed as a percentage of the original odorant solution concentration.

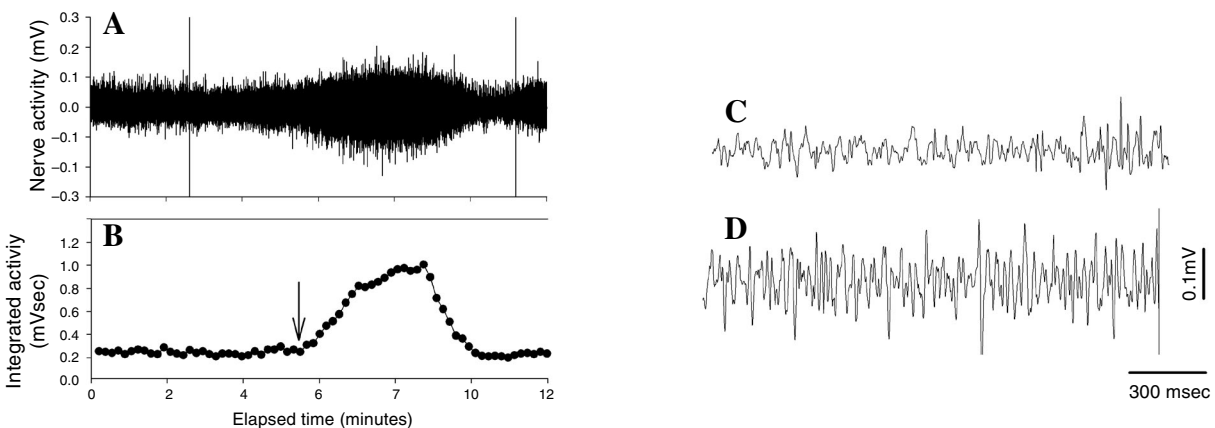
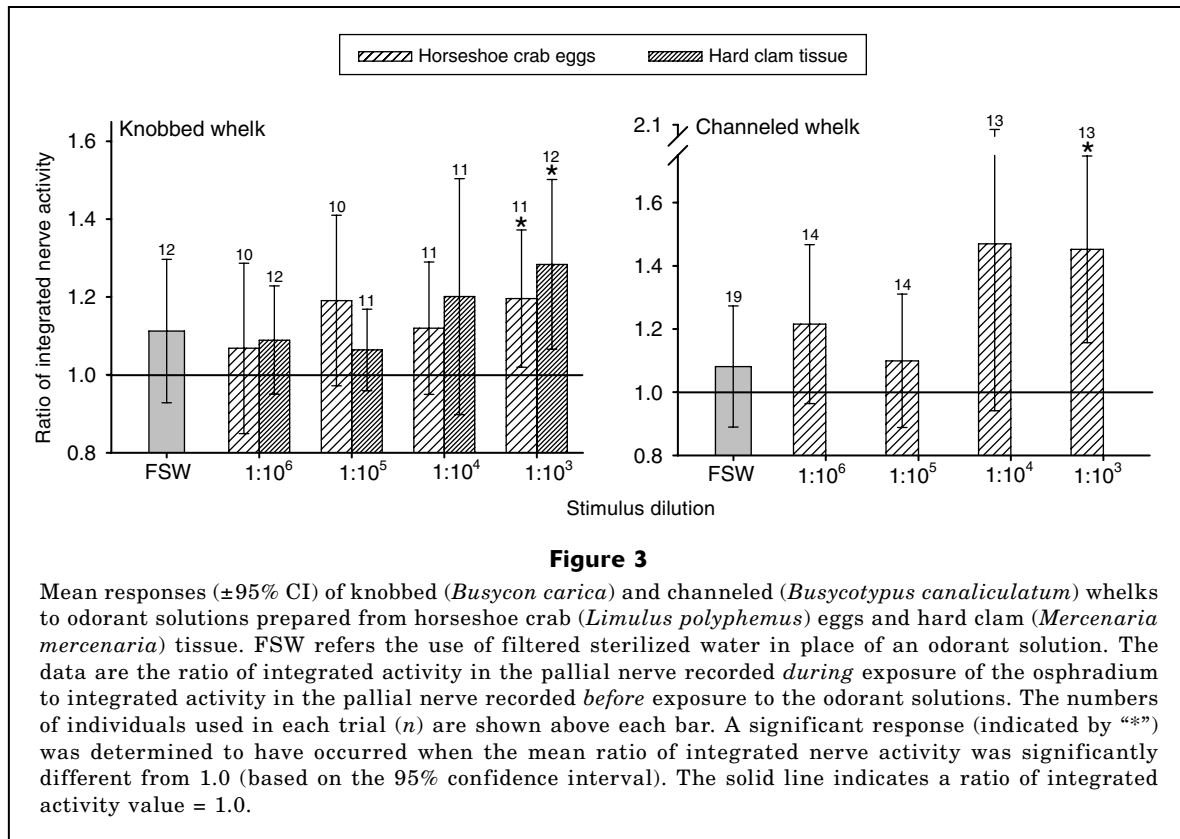


Figure 2

(A) Activity in the pallial nerve in a channeled whelk (*Busycotypus canaliculatum*) before and during exposure of the osphradium to an odorant solution prepared from horseshoe crab (*Limulus polyphemus*) eggs. The arrow in panel B indicates the approximate initial entry of the odorant solution into the osphradium chamber. The two brief large increases in activity (occurring at approximately 2 and 11 minutes after the start of the record) were associated with slight movements of the animal. (B) Nerve activity integrated over 10.2-second intervals was documented with DasyLab 7.0 software (National Instruments Corp., Austin, TX). (C) Nerve activity recorded before initial entry of the odorant solution into the osphradium chamber (3 minutes after the beginning of the record shown in Panel A). (D) Nerve activity at the apex of the response to the odorant solution (8 minutes after the beginning of the record shown in Panel A).

recorded 5 minutes immediately before and 5 minutes immediately after the initial introduction of an odorant solution were analyzed. This procedure resulted in approximately thirty equally spaced data points (i.e., one data point recorded every 10.2 seconds for five minutes),

representing integrated nerve activity in each time period (Fig. 2B). The area under the curves representing integrated nerve activity (Fig. 2) was calculated by summing the points from each 5-minute period (i.e., the data recorded before and during presentation of an



odorant solution). The ratios of nerve activities during application of the odorant solutions to that recorded immediately before application were calculated to test for the presence of responses. This technique reduces the influence of interanimal variability, as well as the inherent variability associated with the use of suction electrodes (Stys et al., 1991).

Data sets were screened for normal distributions with the Anderson-Darling test (Minitab version 14.20.2, Minitab Inc., State College, PA) and all passed. Data were averaged across individuals for each species of whelk, and the standard errors (SE) and 95% confidence intervals (CIs) were calculated. A significant response to an odorant solution was deemed to have occurred if the 95% CI of the mean ratio of integrated nerve activity did not overlap 1.0. This was equivalent to using a two-tailed t -test with $P < 0.05$ taken as the level of significance.

Results

The pallial nerve typically showed spontaneous activity, although nerve activity clearly increased as the concentration of a detectable odorant solution in the osphradium chamber reached maximal concentration (Fig. 2). Once the flow of FSW was resumed (90 seconds after stimulus onset), the concentration of odorant solution in the osphradium chamber decreased (Fig. 1B) and nerve

activity diminished (Fig. 2). Nerve activity generally returned to near prestimulus levels within the recording period (Fig. 2).

Neither species responded to FSW alone (Fig. 3). Odorant solutions extracted from horseshoe crab eggs with FSW elicited a significant increase in pallial nerve activity in both knobbed and channeled whelks at a dilution of $10^3:1$, but not at any of the higher dilution ratios (i.e., lower concentrations) (Fig. 3). Odorant solutions extracted from hard clam tissue also elicited a significant increase in pallial nerve activity of knobbed whelk at the $10^3:1$ dilution ratio, but not at higher dilutions (Fig. 3). This solution was not tested on channeled whelk. In contrast to odorant solutions prepared from horseshoe crab eggs by using FSW, odorant solutions containing horseshoe crab hemolymph generated a species-specific pattern of responses. These elicited significant increases in pallial nerve activity in knobbed whelk at both 100:1 and 133:1 dilutions, whereas channeled whelk did not respond to any of the four concentrations tested (Fig. 4).

Odorant solutions prepared from horseshoe crab eggs by using 50 mM Tris buffer solution were tested only on channeled whelk (Table 1). This stimulus elicited a significant increase in pallial nerve activity at a dilution of $10^3:1$, but not at higher dilutions (Fig. 5)—a result identical to that recorded in channeled whelk with the use of horseshoe crab eggs odorant solutions prepared with FSW (Fig. 3). Channeled whelk did not respond

to the Tris buffer solution alone (Fig. 5). Nor did they respond to either of the molecular weight fractions (>3 kDa nor <3 kDa) prepared from the horseshoe crab egg odorant solutions by ultrafiltration at any concentration tested (Fig. 5). When recombined, the molecular weight fractions again induced a response at $10^3:1$ dilution (Fig 5).

Discussion

Electrophysiological techniques appear suitable for determining odorant solutions that stimulate the pallial nerve in both knobbed and channeled whelks. The sustained increases in nerve activity of the pallial nerves of these two species (exemplified by the results shown in Fig. 2) are similar to those recorded by Bailey and Laverack (1963, 1966) upon exposure of the osphradium of the common whelk (*Buccinum undatum*) to extracts prepared from the blue mussel (*Mytilus edulis*). Bailey and Laverack (1966) also observed extended periods of increased nerve activity, which they termed “the *Mytilus* response.” Wedemeyer and Schild (1995) demonstrated similar increases in activity of the pallial nerves of the pond snail (*Lymnaea stagnalis*) in response to exposure of the osphradium to mixtures of amino acids, to hypercapnia, and to hypoxia. The sustained activity in

the pallial nerve during odor stimulation observed in knobbed and channeled whelks (exemplified in Fig. 2) is also consistent with Ferner and Weissburg’s (2005) findings that slow moving gastropod mollusks locate odor sources in turbulent flow conditions by temporally averaging odor concentrations across a plume. In contrast, the more rapid onset and offset of olfactory responses (i.e., shorter temporal resolution) of the crustacean olfactory system (Gomez and Atema, 1996) appears to hinder their olfactory navigation in turbulent environments (Weissburg and Zimmer-Faust, 1993, 1994).

It is possible that increases in nerve activity in response to odorant solutions also include increases in proprioceptor activity resulting from odor-induced muscular contractions. The pallial nerve appears to be a sensory nerve that conveys a suite of information to the circumesophageal ganglion (Laverack and Bailey, 1963). The pallial nerves of whelks show a low level of almost constant spontaneous activity (Fig. 2C), and rapid onset and offset increases in activity associated with slight spontaneous movements of the head, proboscis, or siphon (Fig. 2A). The origin of the spontaneous activity is unknown, but the latter (the rapid onset and offset increases in activity) are most likely due to activity of axons within the pallial nerve originating from proprioceptors (Laverack and Bailey, 1963). It is possible, therefore, that the ultimate origin for nerve activity seen during application of odorant solutions to the osphradium are proprioceptors, and that these are being stimulated by increased movements in response to application of odorant solutions to the osphradium. This does not, however, invalidate the results. Responses to odorant solutions are still being recorded, albeit not directly in the manner assumed.

One of the objectives of the present project was to determine whether odorant solutions prepared from horseshoe crab eggs stimulated chemosensory receptors in the whelk species targeted by the pot fishery. Ferrari and Targett (2003) suggested the potential use of olfactory attractants from horseshoe crab egg extracts as an alternative to horseshoe crab bait. Their conclusion was based on the behavioral responses of the common mud snail to heat-stable proteinaceous compounds extracted from horseshoe crab eggs. Because both knobbed and channeled whelks respond to horseshoe crab egg odorant solutions prepared with FSW at a $10^3:1$ dilution (Fig. 3), our results support their findings. Horseshoe crab eggs clearly contain a compound (or compounds) detectable by whelks even at low concentrations; therefore odorant solutions prepared from horseshoe crab eggs need to be examined further.

Defining chemical cues from a specific molecular weight class that elicit a response in whelks may prove to be difficult. Channeled whelk responded to odorant solutions prepared from horseshoe crab eggs with Tris buffer solution, as well as the recombined molecular weight fractions (i.e., >3 kDa and <3 kDa). They did not respond to either molecular weight fraction independently (Fig. 5). These results imply that neither the initial extraction (FSW vs. Tris buffer solution) nor ul-

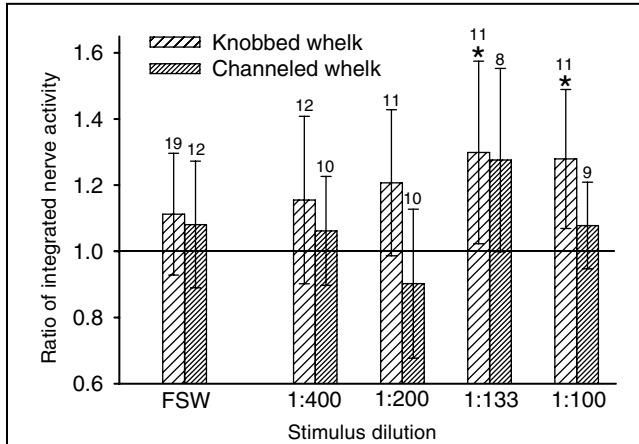


Figure 4

Mean responses ($\pm 95\%$ confidence interval) of knobbed (*Busycon carica*) and channeled (*Busycotypus canaliculatum*) whelks to horseshoe crab (*Limulus polyphemus*) hemolymph diluted with filtered sterilized water. The data are the ratio of integrated activity in the pallial nerve recorded during exposure of the osphradium to odorant solutions to intergrated activity recorded before exposure to odorant solution. The numbers of individuals used in each trial are shown above each bar (n). A significant response (indicated by “*”) was determined to have occurred when the mean ratio of integrated nerve activity was significantly different from 1.0 (based on the 95% CI). The solid line indicates a ratio of integrated activity value = 1.0.

trafiltration procedures destroy the stimulatory components present within the horseshoe crab egg extract. These results also imply that channeled whelk have broadly tuned chemoreceptors requiring a range of low and high molecular weight constituents to elicit a response. Channeled whelk thus appear to differ from other aquatic animals that generally respond to a narrow spectrum of chemical stimulants (e.g., fishes, Carr et al., 1996; Zielinski and Hara, 2007, and crustaceans, Borroni et al., 1986; Kozlowksi et al., 2001). Our results from whelk also differ from behavioral experiments on mud snails, in that mud snails are apparently capable of detecting the low molecular weight fractions (<10 kDa) of horseshoe crab egg extracts prepared by ultrafiltration (Ferrari and Targett, 2003).

Knobbed whelk responded to an odorant solution prepared from hard clam tissue (Fig. 3). Because hard clams are a common prey item of whelks (Magalhaes, 1948; Walker, 1988), we surmise that channeled whelk would respond similarly. Bait based on hard clam tissue, or some extract thereof, could potentially serve as replacement for horseshoe crabs in the whelk pot fishery. The cost of raw material may be prohibitive, however, because hard clams are also harvested for human consumption. Likewise, knobbed whelk responded to horseshoe crab hemolymph even when diluted 133:1 (Fig. 4). Surprisingly, hemolymph did not induce a response in channeled whelk at even the highest concentration tested. There is no apparent explanation for this difference in response by the two species. Horseshoe crab hemolymph may offer some promise as the basis for manufactured bait for the whelk pot fishery primarily because it can be obtained in large quantities from an ecofriendly source (i.e., from the pharmaceutical companies producing LAL), and probably at a reasonable cost. It should be noted that horseshoe crab blood is generally treated with proprietary chemical mixtures during LAL production to prevent clotting and to prepare the amoebocyte cells for further processing. If the chemicals are toxic, this could prevent the use of waste hemolymph directly in manufactured baits and preclude their eventual release into the environment.

In summary, electrophysiological techniques recording afferent activity in the pallial nerves of whelks can be used to identify effective odorant solutions, as well as approximate detectable odor concentration limits. Knobbed and channeled whelks responded to extracts prepared from horseshoe crab eggs, and the former responded to horseshoe crab hemolymph diluted up to 133:1, as well as to extracts prepared from hard clam tissue. Channeled whelk do not respond to individual molecular weight fractions of horseshoe crab

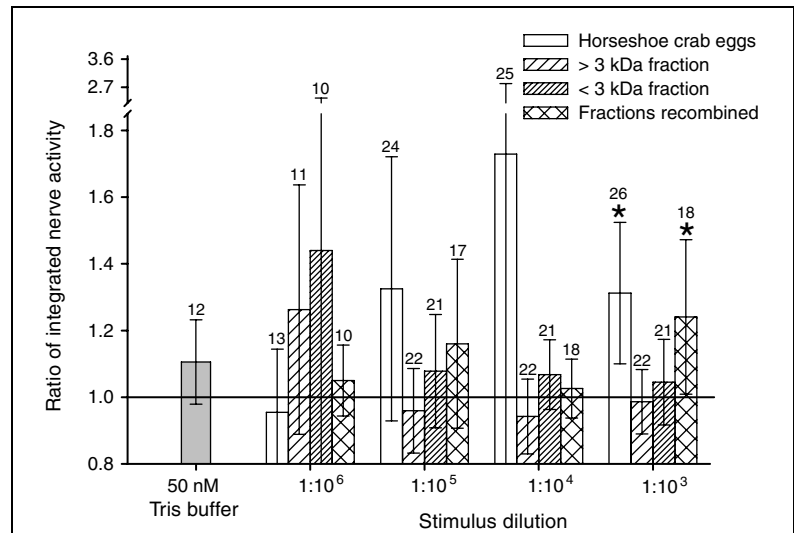


Figure 5

Mean responses ($\pm 95\%$ confidence interval) of channeled whelk (*Busycotypus canaliculatum*) expressed as the ratio of integrated activity in the pallial nerve before the introduction of odorant solutions or 50 mM Tris buffer solution. The odorant solutions were prepared from horseshoe crab (*Limulus polyphemus*) eggs extracted with 50 mM Tris buffer solution, two molecular fractions (>3 kDa and <3 kDa) of this odorant solution prepared by ultrafiltration, and the two molecular weight fractions recombined. The numbers of individuals exposed to various odorant solutions are shown above each bar (n). A significant response (indicated by “*”) was determined to have occurred when the mean ratio of integrated nerve activity was significantly different from 1.0 (based on the 95% CI). The solid line indicates a ratio of integrated activity value = 1.0.

egg extracts, and this result implies that whelks have broadly tuned chemoreceptors and that manufactured baits may need to mimic the complex mixture of odors derived from natural sources.

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